Sim, Hughes, Ashton & McKay
Barristers & Solicitors

Sim & McBurney
Patent & Trade Mark Agents

6th floor, 330 University Avenue
Toronto, Ontario, Canada, M5G 1R7

Tel. (416) 595-1155; Fax (416) 595-1163

DATE: November 6, 1997

Your ref: Our Ref. No.: 1038-422 MIS:ep

TO: **UNITED STATES PATENT AND TRADEMARKS OFFICE**
COMMISSIONER OF PATENTS
Washington, D.C. 20231, U.S.A.

Attn: Examiner Duffy

Telecopier/Fax Number: 1-703-305-7939

Total Number of Pages (Including Cover Sheet): 12

FROM: MICHAEL I. STEWART / Ms. Eren Pyper

COMMENTS: As requested, attached is a copy of the primary reference for U.S. Patent Application Serial Number 08/433,646. Should you require anything further, please advise.

Eren Pyper.

We are transmitting from a Xerox Telecopier 295 - group 3 which is compatible with Groups 1 and 2 and fully automatic on a 24 hour basis. If any part of this message is received poorly or missing please call the telecopier operator immediately at (416) 595-1155.

COLLETTE
OPERATOR

CONFIDENTIALITY NOTICE

FILE

(V)

PROCEEDINGS OF THE FOURTH
INTERNATIONAL SYMPOSIUM
ON

PERTUSSIS

A joint Meeting of the
International Association of Biological Standardization
and the World Health Organization
held at
The Executive Board Room of the World Health Organization
Geneva, Switzerland
25. – 27. Sept. 1984

To the memory of Frank T. Perkins
whose lifelong study of biologics and their control was best
exemplified by his contributions to pertussis vaccine research

148 figures and 184 tables

S. Karger · Basel · München · Paris · London · New York · Sydney

Proceedings of the Fourth International Symposium on Pertussis.
Joint IABS/WHO Meeting, Geneva, Switzerland, 1984
Develop. biol. Standard. Vol. 61, pp. 187-196 (S. Karger, Basel, 1985)

Department of Methodology, National Institute of Public Health,
Geitmyrsveien 75, 0462 Oslo, Norway

[1] Department of Pharmacognosy, Institute of Pharmacy,
University of Oslo, Norway

PURIFICATION AND PRELIMINARY CHARACTERIZATION OF AGGLUTINOGEN 3 FROM *BORDETELLA PERTUSSIS*

J.H. Fredriksen, L.O. Frøholm and B.S. Paulsen[1]

ABSTRACT

One serotype antigen, agglutinogen 3, from *Bordetella pertussis* (strain M2, serotype 1.3), has been purified. The purification procedure included acetone drying of cells harvested from shaking cultures. Agglutinogens were extracted in phosphate buffered saline. Crude extract was heat treated at 80°C for 5 min and precipitated by ammonium sulphate between 25 and 60% saturation at 4°C, providing 50% of the total activity and a five-fold purification. Further purification was attempted by gel filtration chromatography using a TSK-G3000 SW column. The ammonium sulphate precipitated fraction was also separated by anion exchange chromatography using a Mono Q HR 5/5 column. The purification work indicated that agglutinogen 3 is associated with several other substances and that this property can lead to purification difficulties.

The isolation procedure was monitored by an agglutination-inhibition assay. The peak fraction from the ion exchange chromatography was purified up to 27-fold according to the specific activities (inhibition units per mg protein). The yield was only 1% due to severe loss of activity.

In the gel filtration chromatography agglutinogen 3-activity eluted with a maximum activity corresponding to a molecular weight near 450,000. SDS-PAGE analysis indicated that agglutinogen 3 might have a subunit molecular weight of 20,800.

INTRODUCTION

Fourteen agglutinogens have been described for the genus *Bordetella* by other researchers (1, 2). Eight of these have been found in *B. pertussis*. Agglutinogens 1-6 are species specific, while 7 and 13 are shared with *B. parapertussis* and *B. bronchiseptica*. In the 1960's epidemiological data were presented (3) which indicated that the presence of agglutinogen 1, 2 and 3 in vaccines is required for adequate protection against all common serotypes of epidemic strains. Thus the low efficacy of vaccines used in Great Britain in the late 1950's was claimed to be due to a deficiency in agglutinogen 3 content. Based on this, the World Health Organization recommends that vaccines should contain agglutinogen 1, 2 and 3 (4). The role of the agglutinogens in immunity to whooping cough has been a subject of much discussion and investigation, but there is yet no full clarification (5).

The term «agglutinogen» is given to the substances that react with their corresponding antibodies causing *Bordetella* cells to agglutinate. Agglutinogens have been described as membrane protein(s) with molecular weight(s) in the region between 10,000 and 23,000, containing some carbohydrate (6, 7). *B. pertussis* strains

can be divided into serotypes, and normally the classification is done according to occurrence of the three major agglutinogens, which are found in the combinations: 1; 1.2; 1.3; or 1.2.3. Little is known of the nature of the agglutinogens (8).

Previous purification and characterization work has been reported by many other workers (9, 10, 11 and 12). In 1982 others (13) presented evidence for the association of agglutinogen 2 with fimbriae, and showed that the subunit molecular weight was 22,000. Recently data were published which showed that strains of serotype 1.2 and 1.2.3 were strongly fimbriated while 1.3 strains did not have fimbriae (14).

We decided to study agglutinogen 3 more closely, and based our primary investigations in part on the work of another researcher (7).

MATERIALS AND METHODS

Strains of B. pertussis

Strain M2 (serotype 1.3) and strain 360E (serotype 1.2) were obtained from Dr. Noel W. Preston, Pertussis Reference Laboratory, Department of Bacteriology and Virology, University Medical School, Manchester, UK. Strain 28 (serotype 1.2.3), was supplied from Dr. Pavel Novotny, Department of Bacteriology, Wellcome Research Laboratories, Beckenham, UK.

Antisera

Hyperimmune sera were raised in rabbits using an immunizing schedule adopted from others (15) and rendered type-specific by adsorption according to another report (7). Whole cells of serotype 1.3 (strain M2) were used for this immunization. Factor 3 serum was made by adsorption of the 1.3-hyperimmune serum with cells of serotype 1.2 (strain 360E). Hyperimmune serum diluted 1:10 in PBS was incubated with live bacteria at a concentration of 2×10^{10} cells/ml for two hours at 32°C on an orbital shaker. Adsorption was continued overnight at 4°C, and bacteria were removed by centrifugation (3,000 × g for 20 min). The procedure was repeated once with live bacteria and then with autoclaved *B. pertussis* cells, to remove more of the antibodies against LPS and other heat stable antigens.

Growth of bacteria

All strains were stored at -25°C in Greave's solution (16). Growth was initiated on Bordet Gengou plates incubated for 72 hours at 33-34°C and used to inoculate Stainer and Scholte's medium modified by substituting the Tris buffer with disodium glycerophosphate (17). The bacteria were subcultivated ones in Stainer Scholte medium using 250 ml Erlenmeyer flasks containing 50 ml medium. The subculture was grown for 48 hours on an orbital shaker at 140 rpm. The growth from one flask was than used to inoculate 2.8 liter Fernbach flasks containing 1 liter liquid medium. The culture was grown for 48 hours on an orbital shaker at 100 rpm, also at 32°C.

Acetone powder preparation

Bacteria were harvested by centrifugation at 4,800 xg for 20 min using a Sorvall RC-5B Refrigerated Superspeed Centrifuge with a GSA rotor. The cells were resuspended in 13 mM phosphate buffered saline (PBS), pH 7.2, containing 0.123 M NaCl (7). The optical density at 580 nm was measured and the suspension adjusted to about 1.1×10^{12} cells/ml according to the standard opacity reference for pertussis vaccines (18). The ice cold suspension was added slowly, under magnetic stirring, to ten volumes of acetone, pre-cooled to -25°C. Most of the acetone was then decanted and the rest removed by filtration on a Buchner funnel. The precipitate was washed with five volumes of cold acetone followed by two and a half volumes of cold diethyl ether. Residual ether was evaporated and the dry powder was stored in a sealed container at room temperature.

Extraction of surface antigens

The acetone powder was resuspended in PBS by grinding in a mortar followed by homogenizing with a Vortmix- blender (Peter Silver & Sons, England) at 3000 rpm for one minute. Whole cells and larger fragments were removed by centrifugation at 20,000 xg for 30 min. This treatment was repeated twice. The final volume of extract was about 1 ml per 30 mg acetone powder.

Heat treatment of crude extract

The crude extract was heated at 80°C for 5 min and aggregated material removed by centrifugation at 20,000 xg for 30 min.

Ammonium sulphate precipitation

A saturated ammonium sulphate solution at 4°C was added to the supernatant until 25% saturation was reached. After 60 min or more the precipitate was removed by centrifugation (20,000 xg for 30 min). Then the ammonium sulphate concentration was increased to 60% saturation. The precipitate was collected by centrifugation again, and dissolved in PBS diluted 1 : 5 in distilled water. Insoluble material was removed by centrifugation as above. This fraction is called the 25-60% fraction in the following.

Gel filtration chromatography

The 25-60% fraction was fractionated according to molecular weight on a LKB 2135 UltroPac® TSK-Gel 3000 SW column (7.5 x 300 mm), combined with a TSK-GSWP precolumn. The elution buffer was 50 mM phosphate with 0.1 M NaCl, pH 6.7, and the flow rate 0.5 ml/min. Calibration of the column was carried out by using the following standard proteins: Ferritin (MW: 450,000), aldolase (MW: 158,000), ovalbumin (MW: 45,000), and cytochrome c (MW: 12,500), (Combithek® from Boehringer Mannheim, FRG). Blue dextran 2000 (mean MW: 2,000,000) was used to determine the void volume (Pharmacia Fine Chemicals, Uppsala Sweden).

Desalting and buffer-exchange

Before ion exchange chromatography the 25-60% fraction was dialysed against 10 mM ammonium hydrogen carbonate with Spectrapore (Los Angeles, USA) dialysis tubing (cut off 12,000), and then freeze dried. The sample was then dissolved in 30 mM diethanolamine, pH 8.6, centrifuged to remove aggregated material and finally passed through a 0.22 μm Millex-GV® filters (Millipore, Molsheim, France). Ion exchange fractions were desalted by using Sartorius (Göttingen, FRG) collodion bags 132 00 E.

Ion exchange chromatography

The prepacked anion-exchange column, Mono Q HR 5/5® and « FPLC »-equipment (Fast Protein Liquid Chromatography) was from Pharmacia Fine Chemicals (Uppsala, Sweden).

The buffer system used was 30 mM diethanolamine, pH 8.6, with a gradient of increasing NaCl concentration up to 0.35 M NaCl and a flow rate of 1 ml/min. The equipment allowed the slope to be changed. At the end of the chromatography strongly retarded substances were eluted with several injections of 0.5 ml 2 M NaCl.

SDS-PAGE analysis

A Bio-Rad Protean® Dual slab cell was used for SDS-polyacrylamide electrophoresis. The discontinuous buffer-system was used (19). Fifteen per cent acrylamide in 1.5 mm thick gels was used for the separation gel, (and 4% in the stacking gels). The gels were stained with 0.1% Coomassie Brilliant Blue R-250 (20). The following standard proteins were used for molecular weight determination: Phosphorylase A (MW: 94,000), albumin (MW: 67,000), ovalbumin (MW: 43,000), carbonic anhydrase (MW: 30,000), trypsin inhibitor (MW: 20,100) and α-lactalbumin (MW: 14,400), (LMW Calibration Kit, Pharmacia, Uppsala Sweden).

Protein analyses

The protein content of agglutinogen-containing material was determined by the
method of Schaffner and Weissmann (Amidoschwarz 10B staining of proteins), using bovine
serum albumin (Sigma, St. Louis USA) as a standard (21).

Agglutination test

The agglutinating activity of antisera was tested by titration in Titertek ® microplates
(Flow Lab. Irvine United Kingdom), using formaldehyde treated cells of serotype 1.2.3 (strain
28), as standard whole cell antigen in a suspension of 6 x 10[10] cells/ml in PBS with 0.01%
merthiolate (7). To obtain an estimate of the degree of nonspecific agglutination, sera were
also titrated with cells of serotype 1.2 (strain 360E) as whole cell antigen.

Test for liberated agglutinogen

An agglutination-inhibition assay for quantitative determination of the agglutinogen-
content in particle free extracts has been developed (to be published elsewhere). The assay is
based on conventional titration techniques in microplates. Extracts are geometrically diluted
(two-fold), leaving 20 µl in each well. Fifty µl appropriately diluted factor 3 serum is then
added to each well, and after mixing for five minutes, the plate is incubated for one hour at
56°C. (The dilution of the factor serum used in this assay is determined from the agglutination
test mentioned above, and it is one dilution step less than the endpoint in the agglutination
titration). After the first incubation, 50 µl whole cell antigen suspension (serotype 1.2.3, strain
28) is added to each well. The plate is incubated for one more hour and read in a 45° mirror
after standing for about 12 hours or more. The inhibition end point is set at 50% inhibition,
and the agglutinogen activity is expressed in inhibitory units (InU). One InU is the amount of
liberated agglutinogen required to give 50% inhibition in the first well of the dilution row. The
inhibition concentration in the extract tested is determined by dividing the number of units by
the volume of agglutinogen containing extract in a well (i.e. 0.02 ml).

RESULTS

A stable raw material for purification of agglutinogen 3 was obtained by ace-
tone dehydration of bacteria. This acetone powder could be used at least for one
year.

Nearly 30% of the proteins in the crude extract were removed by heat treat-
ment at 80°C for 5 min (Table I). With heating at 80°C for thirty minutes there was
no loss of agglutinogen 3-activity, but there was no further increase in specific activ-
ity either. The next purification step chosen was a ammonium sulphate precipita-
tion. Half of the agglutinogen 3-activity was obtained in the 25-60% fraction (Table
I). The purification was five-fold at this stage.

Further purification was attempted by using gel filtration and ion exchange
chromatography with several kinds of column material. Fig. 1 shows a gel filtration
chromatogram from a fractionation on a TSK-G3000 SW column. In this experi-
ment 4.5 mg protein and an activity of 1.28 x 10 InU was applied by a 0.5 ml loop.
On this analytical column the agglutinogen 3-activity (stippled line) eluted with a
maximum activity corresponding to MW 450,000. The agglutinogen 3-activity had
an extended distribution ranging from the void volume to MW 45,000. The 1 ml
fraction with maximum activity (800 InU/ml) had a specific activity of 6 x 10[4]
InU/mg protein, which was calculated to be a seven-fold purification over all. The
main fraction (7-10 ml) corresponded to 12% of the activity applied. Attempts to
pool fractions from the TSK-G3000 SW column, or use preparative gel filtration
columns, to obtain more material for further purification were unsuccessful. The
decrease in specific activity was considerable. On a Ultrogel AcA 22 (approximate
exclusion limit : MW 1,200,000) there was no activity in the void volume, the acti-
vity eluted corresponding to MW 450,000, but the resolution was less than on the

Table I. Purification of agglutinogen 3

Step	Volume (ml)	Protein (mg/ml)	Activity (InU/ml×10^3)	Specific activity (InU/mg prot.×10^3)	Yield (%)	Purification
Crude extract	395	3.39	3.2	0.9	100	1.0
Heat treated extract	395	2.40	3.2	1.3	100	1.4
The 25-60% fraction	13	10.68	51.2	5	53	5
Peak fraction from anion exchange chrom.	13*	0.03	0.8	27	1	27

* Extrapolated from the 0.5 ml which was actually fractionated in this experiment.

TSK-G3000 SW column. Using a Sephacryl S-200 gel (approximately exclusion limit: MW 250,000) the activity eluted in the void volume (the buffer was the same as for the analytical column).

Fractionation with use of a Mono Q-anion exchange column also produced separation difficulties. Fig. 2 shows a typical chromatogram from an anion exchange separation. The activity in the 0.5 ml desalted 26-60% fraction applied was 1.28 × 10^4 InU, and the protein amount was 5 mg. Agglutinogen 3-activity (stippled line) was found in two major peaks both with an activity of 800 InU/ml, and also at a level of 200 or 100 InU/ml over most of the chromatogram. The first activity peak came in mixture with other non-retarded substances. Retarded agglutinogen 3-activity eluted with maximum specific activity near 0.06 M NaCl. This second activity peak appeared slightly before a distinct protein peak. The rest of the retarded agglutinogen 3-activity seemed to follow the main protein distribution. The activity peak fraction had a specific activity of 2.7 × 10^4 InU/mg protein, which gives a 27-fold purification (Table I). The total yield was 1%. Anion exchange chromatography was also attempted at pH 9.2 but this higher pH gave a chromatogram where the activity peak in the void was the same, while the retarded agglutinogen 3-activity peak was more retarded and more contaminated with other components.

Fig. 3 shows the SDS-PAGE patterns from different steps in the purification procedure. Whole cells, crude extract, heat treated extract and the two ammonium sulphate precipitated fractions show complex patterns of 30-40 bands (lanes 1-6).

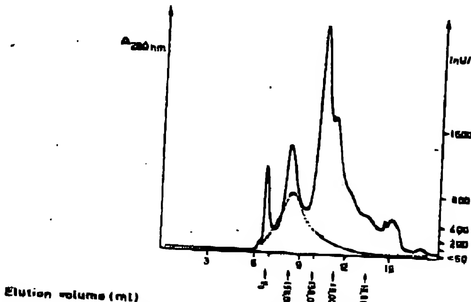

Fig. 1. Gel filtration chromatography of ammonium sulphate precipitated agglutinogen 3 on a TSK-G3000 SW column. The elutionbuffer was 50 mM phosphate with 0.1 M NaCl, pH 6.7. Flow rate was 0.5 ml/min and fraction size 0.5 ml. Protein concentration (——) was monitored at 280 nm and agglutinogen 3-activity (·-·-·-) monitored by agglutination-inhibition. Elution properties of standard proteins and blue dextran 2000, used for calibration of the column, are indicated by arrows.

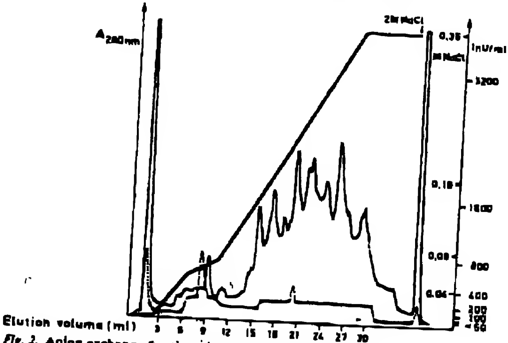

Fig. 2. Anion exchange fractionation of ammonium sulphate precipitated agglutinogen 3 on a Mono Q column. The buffer was 30 mM diethanolamine, pH 8.6. Retarded material was eluted by a linear gradient of up to 0.35 M NaCl with different slopes. The flow was 1 ml/min and fraction size 0.5 ml. Retarded agglutinogen eluted with maximum specific activity near 0.06 M NaCl.

Fig. 3. SDS-PAGE separation of material from different steps in the purification of agglutinogen 3. Sample loads in the different lanes given in parenthesis. Lane 1, whole cells (8 µg); lane 2, crude extract (10 µg); lane 3, heat treated extract (7 µg); lane 4, precipitate at 25% saturated ammonium sulphate (7 µg); lane 5, precipitate between 25-60% saturation (23 µg); lane 7, activity peak from anion exchange fractionation (3 µg); lane 8, standard proteins (see materials and methods).

The amount of protein applied varied from 3 to 23 µg (see legend, Fig. 3). There are no striking differences between the patterns in lanes 1-6. Lane 7 which contains agglutinogen 3 after anion exchange fractionation shows one major band with a molecular weight of 20,800. Some additional weak bands were seen in the 10,000 and 30,000 molecular weight regions.

DISCUSSION

The investigation presented in this paper illustrates factors which are important for purifying agglutinogen 3 and deal with the exact nature of this substance. The preparation of an acetone powder of bacteria proved a good starting material for preparing agglutinogen 3 containing extracts. In this form the biomass is easily stored before extraction and purification. Agglutinogen 3 is stable to acetone and diethylether, thus it is possible to remove different kind of lipids at an early stage in the purification procedure. The simple extraction procedure described gives a high yield of agglutinogen 3 in the crude extract. Our preparation procedure differs from the method of others (7,22) who used mechanical disintegration, a method reported to be gentle and perhaps more suitable for several kinds of substances from *B. pertussis* cells.

Heat treatment at 80°C of the crude extract seems to be advantageous. Agglutinogen 3 is sufficiently heat resistant and one gets rid of 30% of the protein present. The heat resistance is in accordance with reports in the literature (8). The ammonium sulphate precipitation showed that agglutinogen 3 did not precipitate within a narrow concentration range. It seems likely that this difficulty reflected a tendency of agglutinogen 3 to adsorb to other substances. The 25-60% fraction contained about 50% of the activity, but such a precipitate will also contain many contaminating substances.

Gel filtration chromatography as well as anion exchange chromatography, also suggested that agglutinogen 3 is associated with several substances. Gel filtration studies showed that elution properties of agglutinogen 3-activity corresponded to a molecular weight near 450,000. A high-resolution method, anion exchange with a Mono Q column, gave a total purification of up to 27-fold, but a disturbing low yield of 1% and may reflect the adsorption or aggregation properties reported previously. Agglutinogen 3 did not move in the starch bloc electrophoresis used by other workers (23). It has been observed that several fractions from a Sephacryl S-300 column all induced agglutinin production when injected in mice (24). Altogether the data indicate that agglutinogen 3 is difficult to purify.

The SDS-PAGE analysis of crude fractions gave a complex pattern as also found by other investigators (25). The major stained band with a molecular weight of 20,800, detected in the most purified fraction from anion exchange chromatography, might be agglutinogen 3. The molecular weight of the band is in agreement with previous reports (6, 13), but its identity would have to be verified by use of specific immunological techniques or further purification.

The degree of purification of agglutinogen 3 is probably greater than 27-fold. This belief is based on the anion exchange chromatogram and the slab-gel results. There was little material in the region where the activity peak of retarded agglutinogen 3 eluted, and in SDS-PAGE there was just one major band left. The agglutination-inhibition assay might not give a correct value for purified fractions since agglutinogen 3 aggregates and this may result in fewer exposed reactive sites.

Attempted purification steps after the ammonium sulphate precipitation were unsuccessful with respect to developing a preparative procedure. Some useful information on the properties of agglutinogen 3 were obtained which can lead to development of a separation procedure with good yield. The procedure as reported in this paper could be used to obtain more purified material for stability studies of agglutinogen 3, for immunoblot and other investigations. At present we have not detected any denaturation problems in crude extracts and ammonium sulphate precipitated fractions. This may be due to protecting substances with which agglutinogen 3 is associated in the earlier steps. Further success with the purification seems to require a suitable stabilizing agent or possibily a detergent. Besides indicating methods which can lead to development of a better purification procedure for agglutinogen 3, this paper also demonstrates problems which arise when working with this substance.

ACKNOWLEDGEMENTS

Thanks are due to Professor Jens Kristian Wold, Institute of Pharmacy University of Oslo, for access to the Pharmacia FPLC system and the UltroPac™ column and to cand. pharm. Svein Haavik for help and discussions.

The investigation was supported by grants from Directorate of Health Pharmaceutical Department – Norway, Norsk Farmaceutisk Selskaps Stipendiefond, Pasteurlegatet and the University of Oslo.

REFERENCES

1. Andersen E.K. Serological studies on *H. pertussis, H. parapertussis* and *H. bronchisepticus*. Acta. Path. Microbiol. Scand. 1953 ; 33 : 202-224.
2. Eldering G., Hornbeck C., Baker J. Serological studies of *Bordetella pertussis* and related species. J. Bact. 1957 ; 74 : 133-136.
3. Preston N.W. Type-specific immunity against whooping-cough. Br. Med. J. 1963 ; ii : 724-726.
4. World Health Organization, Expert Committee on Biological Standardization. Requirements for pertussis vaccine. Thirtieth Report. WHO. Tec. Rep. Ser. no 638. Geneva 1979 ; 60-80.
5. Wardlaw A.C., Parton R. Pertussis vaccine. In : Easmon C.S.F. and Jeljaszewicz J., eds. Medical Microbiology. London : Acad. Press 1983 : 2 : 207-253.
6. Zakharova M.S. Theoretical outlines on the preparation of a noncellular pertussis vaccine. In : Manclark C.R., Hill J.C., eds. International Symposium on Pertussis. Washington DC : US DHEW Publ. no (NIH) 79-1830. 1979 : 320-326.
7. Novotny P., Cownly K. Effect of growth conditions on the composition and stability of the outer membrane of *Bordetella pertussis*. In : Manclark C.R., Hill S.C., eds. International Symposium on Pertussis., Washington DC : USDHW Publ. no (NIH) 19-1830. 1979 ; 99-123.
8. Munoz J.J., Bergman R.K. *Bordetella pertussis* : Immunological and other biological activities. In : Rose N., ed. Immunological series. New York : Marcel Dekker, 1977 ; 4.
9. Onoue K., Kitagawa M., Yamamura Y. Chemical studies on cellular components of *Bordetella pertussis*. J. Bact. 1961 ; 82 : 628-656.
10. Nakase Y., Kasuga T. Purified K-agglutinogen of *Bordetella pertussis* and its properties. Jpn J. microbiol. 1971 ; 15 : 247-256.
11. Denisova T.P., Obtaining highly purified *B. pertussis* agglutinogen. Zh. Microbiol. Epidemiol. Immunol. 1969 ; (5) : 97-101.
12. Zulina L.V., Zakharova M.S., Bagdasarova I.P. Obtaining and chemical characteristics of agglutinogen with the properties of factor 3. Zh. Microbiol. Epidemiol. Immunol. 1972 : (3) : 101-104.
13. Ashworth L.A.E., Irons L.I., Dowsett A.B. Antigenic relationship between serotype-specific agglutinogen and fimbriae of *Bordetella pertussis*. Infect. Immun. 1982 ; 37 : 1278-1281.
14. Carter E.J., Preston N.W. Association between *Bordetella pertussis* agglutinogen 2 and fimbriae. J. Med. Microbiol. 1984 ; 18 : 87-94.
15. Harboe N., Ingild A. Immunization, isolation of immunoglobulins, estimation of antibody titre. In : Axelsen N.H., Kroll J. and Weeke B., eds. Quantitative immunoelectrophoresis. Oslo : Universitetsforlaget. 1973 ; 161-169.
16. Craven D.E., Frash C.E., Robbins J.B., Feldman H.A. Serogroup identification of *Neisseria meningitidis* : Comparison of an antiserum agar method with bacterial slide agglutination. J. Clin. Microbiol. 1978 : 7 : 410-414.
17. Lothe R.A., Froholm L.O., Westre G., Kjennerud U. Stainer and Scholte's pertussis medium with an alternative buffer. J. Biol. Stand. 1984 ; 13 :
18. Csizer Z., Szammet H., Niedermayer N.N., Zsidai J., Auber E., Joo I. Estimation of bacterial mass of pertussis vaccine by opacity and dry weight determination. J. Biol. Stand. 1977 ; 5 ; 289-295.
19. Laemmli U.K. Cleavage of structual proteins during the assembly of the head of bacteriophage *T4*. Nature 1970 ; 227 : 680-685.
20. Nicolas R.H., Goodwin G.H. In : Johns E.W., ed. The HMG chromosomal proteins. Acad. Press 1982 ; 41-68.
21. Schaffner W., Weissmann C. A rapid, sensitive, and specific method for determination of protein in dilute solution. Anal. Biochem. 1973 ; 56 : 502-514.

22. Novotny P. A simple rotary disintegrator for microorganisms and animal tissues. Nature 1964 ; 202 : 364-366.
23. Ross R.F., Munoz J. Antigens of *Bordetella pertussis*. V. Separation of agglutinogen 1 and mouse-protective antigen. Infect. Immun. 1971 ; 3 : 243-248.
24. Askelöf P., Granström M., Gillenius P., Lindeberg A.A. Purification and characterization of a fimbrial hemagglutinin from *Bordetella pertussis* for use in an enzyme-linked immunosorbent assay. J. Med. Microbiol. 1982 ; 15 : 73-83.
25. Parton R, Wardlaw AC. Cell-envelope proteins of *Bordetella pertussis*. J Med Microbiol 1975 : 8 :47-57.